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NOTE

Detection of Lymphotropic Herpesviruses by Multiplex Polymerase Chain Reaction

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Human lymphotropic herpesvirus is known to be a major pathogen associated with various diseases in bone marrow transplantation (BMT) recipients. A multiplex nested-polymerase chain reaction (PCR) method was developed for the simultaneous detection of human lymphotropic herpesviruses, including Ebstein-Barr virus (EBV), cytomegalovirus (CMV), and human herpesvirus 6 variants A and B (HHV6-A, HHV6-B). To demonstrate the usefulness of multiplex PCR for the analysis of clinical samples, peripheral blood mononuclear cells and serum from BMT recipients were analysed. The results showed that a clear detection could be made between EBV, HCMV and HHV-6. This multiplex PCR assay is an efficient and cost-effective approach to the analysis of large numbers of samples to determine the epidemiological importance of EBV, HCMV and HHV-6.

Key words: multiplex nested-polymerase chain reaction (PCR), human lymphotropic herpesvirus, bone marrow transplantation (BMT)

Human lymphotropic herpesviruses, including Epstein-Barr virus (EBV), cytomegalovirus (CMV), and the most recently discovered human herpesvirus 6, variants A and B (HHV6-A, HHV6-B), are responsible for a wide variety of human diseases. Their infections are a major cause of morbidity and mortality among immunocompromised patients, caused either by primary infection or by reactivation in immunosuppressive conditions (1, 9, 13). Some of these herpesviral diseases are undoubtedly associated with more than one virus. In most clinical syndromes of herpesviral etiology, different herpesviruses are most often involved, requiring differential diagnosis not only for epidemiological purposes, but also because of the need for treatment with specific antiviral drugs (5). Until recently, the etiological diagnosis of herpesviral infections relied solely on serological tests or virus isolation in cell cultures for each of these viruses. Over the last few years, the amplification of viral DNA by polymerase chain reaction (PCR) has become one of the most widely used techniques in human diagnostic virology. PCR amplification has recently been used as a primary modality to diagnose EBV, CMV, and HHV6-A and B infection, particularly in immunosuppressed patients. Direct viral detection by PCR has advantages over other diagnostic methods. Viral culture for certain herpesviruses, such as EBV, and HHV-6, is technically difficult and not done in routine diagnostic laboratories. Serologic responses, frequently used to diagnose viral infection in immunocompetent patients, are not reliable in immunosuppressed patients (12). Whereas multiplex PCR is a modification of the original PCR procedure, it allows the simultaneous amplification of DNA from different pathogens by the use of several primer pairs in the same reaction (2). Some advantages of the multiplex PCR methods include reduced labor intensiveness, and the economy of both test sample and reagents compared to single analytical PCRs.

We attempted to develop a convenient and reliable multiplex PCR method to detect human lymphotropic herpesviruses, such as EBV, CMV and HHV-6, with simultaneous amplification. The technique we developed in this study could avoid the need to test clinical specimens separately for each lymphotropic herpesvirus.

Prototype viral strains EBV B95-8 (ATCC VRL-1612), CMV AD169 (ATCC VR-538), and HHV6-B strain (HST

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strain; kindly provided by K Tanaka-Taya, Department of Developmental Medicine Pediatrics, Osaka University Graduate School of Medicine, Osaka, Japan) were used as positive controls. Template DNAs for the different viruses were isolated from each prototype strain.

DNA was isolated from 200 μl of each virus strain by use of a QIAamp DNA mini kit (QIAGEN, Germany). Precautions were taken to prevent contamination, as described by others (8). PCR primers for EBV, HCMV and HHV-6 were described previously by others (3, 7, 10). The sizes of amplification products of EBV, HCMV, and HHV-6 were 129 bp, 230 bp, and 421 bp, respectively. Each 5 μl of the DNA extract was used as the template DNA for multiplex PCR. A 50 μl PCR mixture consisted of 10 pmol of each primer and 2 mM each of four deoxynucleoside triphophates (Roche, Germany) in 10× PCR buffer (50 mmol/L KCl, 1 5 mM $MgCl_2$, Roche), 0.1% BSA (Takara, Japan) and 5 U of Taq DNA polymerase (Roche). Reaction mixtures were given an initial denaturation step at 94°C for 2 min and followed by 30 cycles, consisting of 30 sec at 94°C, 1 min at 53°C, and 30 sec at 72°C. A final extension step at 72°C was carried out for 5 min. These cycling conditions were optimized for use with a Gene Cycler™ (BIO-RAD, Hercules, CA, USA). After the first amplification round, 2 μl of the reaction product was added to 48 μl of a second reaction mixture consisting of 10 pmol each primer and 2 mM each of four deoxynucleoside triphophates, in 10 × PCR buffer (50 mmol/l KCl, 15 mM $MgCl_2$), 0.1% BSA, and 5 U of Taq DNA polymerase. Amplification was performed under the same conditions used for the first round. The negative controls consisted of Jurkat cell DNA extracts coupled with distilled water that were interspersed within assays to ensure that no cross-contamination occurred during pipetting. To avoid a false positive PCR result by carry-over contamination, aliquoting of clinical specimens, preparation of reagents, DNA extraction, first round amplification, and nested-PCR were carried out in safety cabinets located in separate laboratories, all of them far away from the area where amplified products were analyzed. A 2% agarose gel containing 5 μg/ml ethidium bromide was used to stain and visualize PCR products.

The shared clinical features of lymphotropic herpesviruses and the increased use of PCR methods in their diagnosis led us to develop a multiplex nested-PCR assay for simultaneous detection of EBV, CMV, HHV6-A and HHV6-B in a single assay (4, 6). One of the major problems with multiplex PCR methods is to make compatible the oligonucleotide primers mixed in the PCR reaction. Each reaction component and all thermal cycling parameters were thoroughly standardized (2).

The specific bands obtained from each standard control herpesvirus after two rounds of amplification, resulted in DNA fragments of 129 bp for EBV, 230 bp CMV and 421 bp HHV6-B as shown in Fig. 1 (Lanes 5, 6 and 7).

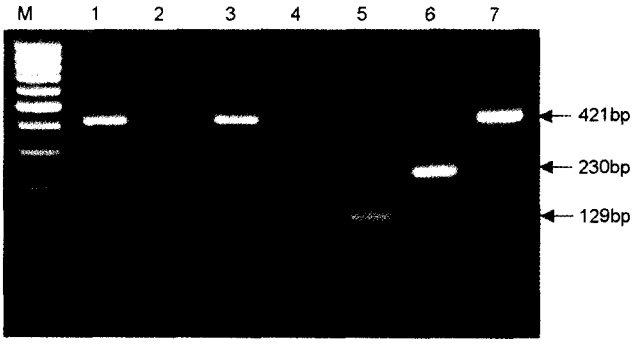

Fig. 1. Agarose gel showing the specific bands obtained for each herpesvirus after two rounds of amplification. M : 100 bp DNA marker Lane 1-4 : clinical samples, Lane 5 : EBV, Lane 6 : HCMV, Lane 7 : HHV6-B

Non-specific amplifications were not detected. Results obtained with clinical specimens are also shown in Fig. 1 (Lane 1, 2, 3 and 4). Specific HHV-6B DNA were detected in two cerebrospinal fluid samples from patients suffering from post-BMT encephalopathy.

The results of the multiplex PCR assay after testing clinical specimens confirmed our expectations. CSF samples were considered appropriate specimens for HHV-6 detection, as this virus often gives rise to neurological disorders. DNA from EBV, CMV and HHV-6 were also detected in serum from transplant recipients and children with infectious mononucleosis, and in urine from infants with congenital CMV infection (5). Viral diagnosis for all these specimens had been previously established by serologic tests, shell-vial culture, pp65 antigenemia or an alternative PCR method (Table 1). Using an internal control, consisting of 100 molecules of a cloned and purified genome fragment of pesudorabies herpesvirus (PrV) DNA, was included in the DNA extraction buffer at the beginning of the process, to detect false negative results (11).

In summary, we think that the multiplex PCR method described here has an extensive spectrum of potential application in clinical settings in which more than one lymphotropic herpesvirus could be involved. EBV, CMV and HHV-6 could be detected and characterized by using a single assay, avoiding the need to test clinical specimens with separate PCR methods. In addition to its use for large scale analyses, multiplex PCR may have other clinical applications. The major problem with the use of multiplex PCR in specimens from immunocompromised patients is the relationship of positive results with clinical disease.

Table 1. The results of multiplex PCR in bone marrow transplantation (No of samples = 200)

Sample	EBV	HCMV	HHV-6	HCMV+HHV-6
Serum DNA	0/200	17/200	19/200	3/200
PBMC DNA	1/200	37/200	49/200	7/200

PBMC : Peripheral Blood Mononuclear Cell

This is particularly difficult with herpesviruses, especially since positive PCR results may not always be accompanied by signs or symptoms in the patient. This can leave the clinician in a dilemma of whether or not to treat. Two approaches have been used to address this problem. Quantitative PCR can be used to measure the viral load with the expectation that a high viral load is likely to herald clinical disease. The other approach is to test sequential specimens and only recommend treatment if positive results persist rather than responding to what may be a transient reactivation of a herpesvirus. Multiplex PCR may be readily applied in the latter situation, but multiplex quantitative PCR remains a formidable technological challenge.

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